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14. ABSTRACT

The investigators successfully performed initial control experiments in unsensitized animals applying the proposed induction and maintenance immunosuppressive regimen to assess its safety and feasibility for maintaining VCA allograft survival in this model. Successful VCA (i.e. swine heterotopic hind limb) transplantation was performed across a full SLA mismatch from a CC donor to an AD recipient animal. The animal was treated with non-myeloablative total body irradiation and thymic irradiation two days prior to transplantation and with continues calcineurin inhibitor-based immunosuppressive maintenance therapy using tacrolimus at target trough levels of 10-20 ng/ml for 30 days.

15. SUBJECT TERMS
vascularized composite allotransplantation, sensitization, autologous hematopoietic stem cell transplantation, antibody mediated rejection, donor specific antibodies

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PI: Gerald Brandacher, MD

1. INTRODUCTION

For many devastating combat injuries where conventional reconstruction is not possible, Vascularized Composite Allotransplantation (VCA) has become a viable alternative. This approach provides new, exciting options for Wounded Warriors that could restore appearance, anatomy, and function better than other available treatment options. However, clinical management of these injuries prior to transplantation frequently requires multiple blood transfusion or skin grafts resulting in the formation of alloantibodies (anti-HLA IgG Abs) and sensitization. In solid organ transplantation (SOT), such pre-sensitization is the greatest risk factor for allograft rejection and long-term graft failure, and causes patients to be excluded as candidates for transplantation. However, the role of donor-specific antibodies (DSA) and mechanisms of antibody-mediated rejection (AMR) in VCA are largely unknown. Thus, there is an imminent need to develop a better understanding of the mechanisms related to DSA and AMR after VCA as well as to implement novel clinically relevant desensitization protocols that would be applicable to a cadaveric donor setting.

The objective of this project therefore is to comprehensively investigate the mechanisms and impact of pre-existing and de-novo DSA and AMR in VCA and to develop a clinically relevant desensitization protocol that will subsequently broaden the population of sensitized patients eligible for reconstructive transplantation. The investigators will test their central hypothesis that that the impact and mechanisms, of AMR in reconstructive transplantation as well as the cadaveric donor setting will require specifically tailored desensitization strategies and treatment regimens in order to improve access and outcomes for highly sensitized VCA candidates in a pre-clinical large animal model.

2. KEYWORDS

vascularized composite allotransplantation, sensitization, autologous hematopoietic stem cell transplantation, antibody mediated rejection, donor specific antibodies

3. ACCOMPLISHMENTS

The group obtained approval for the proposed project from both the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University as well as the Animal Care and Use Review Office (ACURO) of the Department of Defense.

During year one of this project successful VCA (i.e. swine heterotopic hind limb) transplantation was performed across a full SLA mismatch from a CC donor to an AD recipient animal. The animal was treated with non-myeloablative total body irradiation and thymic irradiation two days prior to transplantation and with continues calcineurin inhibitor-based immunosuppressive maintenance therapy using tacrolimus at target trough levels of 10-20 ng/ml for 30 days.

A. Major Goals

The major goals of this project for Year 1 as stated in the approved SOW were:

Major Task 1: Identify the role of presensitization, DSA, and mechanisms of AMR in VCA

- Subtask 1.1- Receive Institutional Animal Care and Use Committee (IACUC) and DoD Animal Care and Use Review Office (ACURO) approval
- Subtask 1.2 - Perform swine heterotopic hind limb transplantation (Groups 1, 2); monitor kinetics of circulating DSA (flow cytometry) in sensitized recipients after VCA, and characterize DSA (IgG) and C3d/C4d deposition (immunofluorescence) in tissue components of VCA
- Subtask 1.3- Evaluate histopathological changes (H&E) and inflammatory infiltration (IHC or IF for CD3, CD4 and CD8 (T cells), ED1 (circulating macrophages), ED2 (resident macrophages), CD19 (B cells) in pre-sensitized VCA recipients

Major Task 2: Identify the role of de-novo DSA and impact on AMR in VCA

- Subtask 2.1- Perform swine heterotopic hind limb transplantation (Group 3), monitor kinetics of circulating DSA (flow cytometry) in sensitized recipients after VCA, and characterize DSA (rat IgG) and intragraft C3d/C4d deposition (immunofluorescence)

Table 1: Progress against the SOW

Task	**Start Date**	**End Date**	**% Complete**	**Comments**
Subtask 1.1	September 2016	February 2017	100	
Subtask 1.2	February 2017	May 2017	33%	Due to the lack of available animals for transplant from the vendor at MGH/Columbia progress has been delayed
Subtask 1.3	May 2017	July 2017	33%	Due to the lack of available animals for transplant from the vendor at MGH/Columbia progress has been delayed
Subtask 2.1	July 2017	August 2017	0%	Due to the lack of available animals for transplant from the vendor at MGH/Columbia progress has been delayed

B. Accomplishment of Goals

<u>Subtask 1.1- Receive Institutional Animal Care and Use Committee (IACUC) and DoD Animal Care and Use Review Office (ACURO) approval</u>

The major activity under Task 1A was to establish an IACUC and ACURO approved animal research protocol. The specific objectives of this task were met by the approval of both the IACUC and the ACURO protocols, allowing the VCA Laboratory to perform the proposed in-vivo transplantation experiments.

Subtask 1.2 - Perform swine heterotopic hind limb transplantation (Groups 1, 2); monitor kinetics of circulating DSA (flow cytometry) in sensitized recipients after VCA, and characterize DSA (IgG) and C3d/C4d deposition (immunofluorescence) in tissue components of VCA

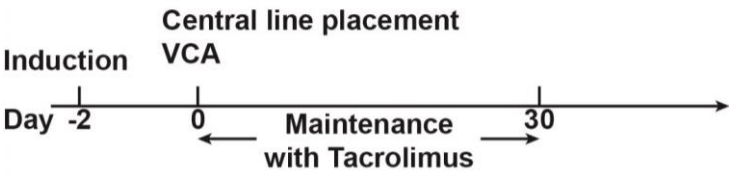

Figure 1: Treatment regimen of the recipient animal

Successful VCA (i.e. swine heterotopic hind limb) transplantation was performed across a full SLA mismatch from a CC donor to an AD recipient animal. The animal was treated with non-myeloablative total body irradiation and thymic irradiation two days prior to transplantation and with continues calcineurin inhibitor-based immunosuppressive maintenance therapy using tacrolimus at target trough levels of 10-20 ng/ml for 30 days (**Figure 1**).

Furthermore, on the day of transplantation a central venous access catheter was placed to allow for continuous treatment as well sampling of whole blood to obtain peripheral blood mononuclear cells (PBMCs) and serum. Animals were monitored clinically and photo documentation of the VCA allograft was performed (**Figure 2**).

Prior to treatment (baseline) and at various

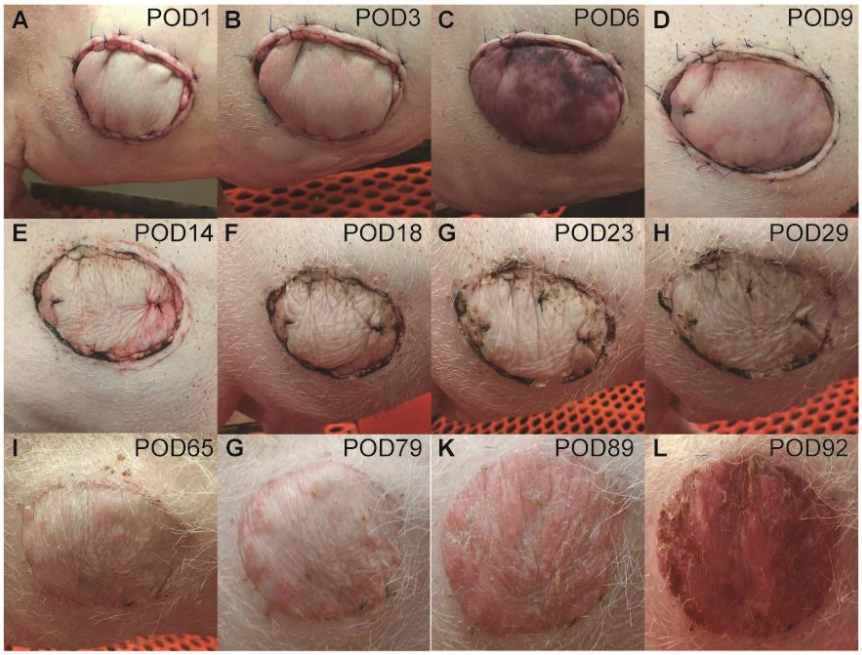

Figure 2: Clinical photos of skin component of VCA graft

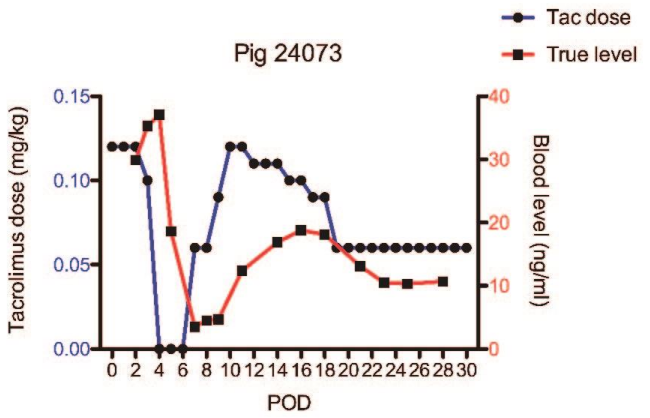

Figure 3: Tacrolimus dose and trough blood levels

time points following transplantation on post-operative days (POD) 0, 1, 3, 5, 7, 10, 14, 21, 30 serum and PBMCs were harvested and stored for further downstream analysis of cellular and humoral markers of allograft rejection and maintenance.

Figure 3 outlines dynamics of tacrolimus dose and monitored blood levels over the course of 30 days of treatment indicating sufficient immunosuppression using target tacrolimus trough levels of 10-20 ng/ml.

Furthermore, to determine baseline alloreactivity amongst donor and recipient we performed in-vitro mixed lymphocyte reactions (**Figure 4**) indicating a viable donor-specific proliferative response of recipient CD4 positive T cells.

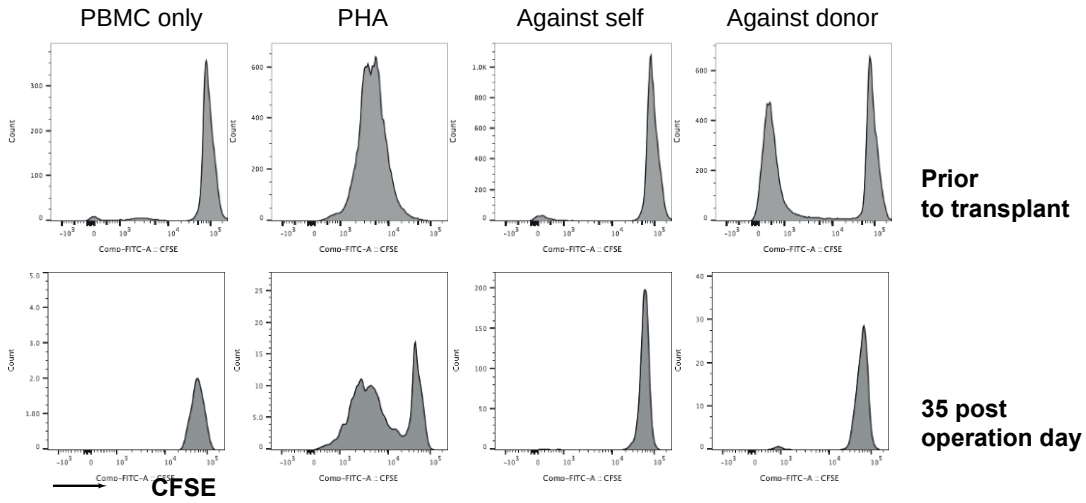

Figure 4: Mixed lymphocyte reaction (MLR) showing donor hypo-responsiveness

<u>Subtask 1.3- Evaluate histopathological changes (H&E) and inflammatory infiltration (IHC or IF for CD3, CD4 and CD8 (T cells), ED1 (circulating macrophages), ED2 (resident macrophages), CD19 (B cells) in pre-sensitized VCA recipients</u>

Skin biopsies were taken on POD 0, 7, 14, 30 and fixed in 10% PFA and embedded in paraffin for downstream analysis (**Figure 5**). **Figure 6** shows examples of immunohistochemistry stainings to assess CD3 T cells, CD20 B cells, and Foxp3 regulatory T cell infiltration in the skin component obtained with the protocols that were established by the investigators. Furthermore, assays to investigate and quantitatively analyze the repopulation of T cells, B cells, and Mono/Granulocytes were established over the past several by using antibody staining and flow cytometry. In addition,

assays to detect donor derived cells using Porcine Allelic Antigen (PAA) were optimized during this reporting period (**Figure 7**).

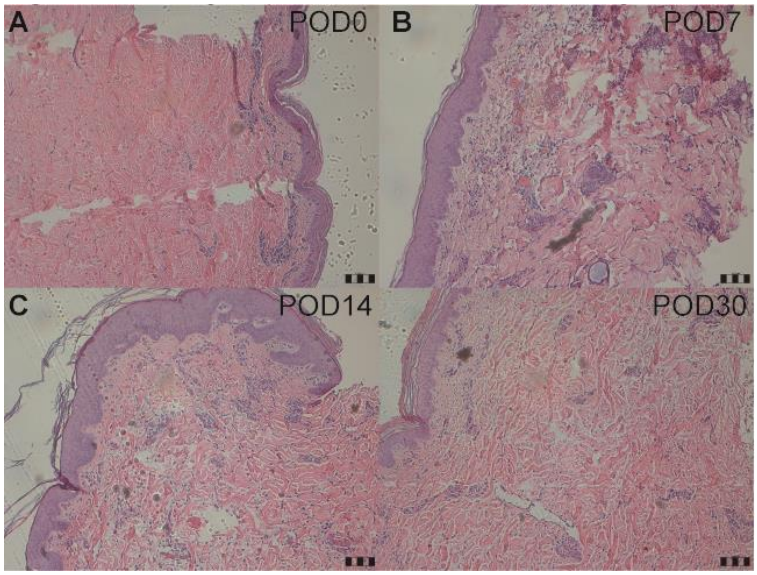

Figure 5: Histological photos of skin biopsy in VCA graft

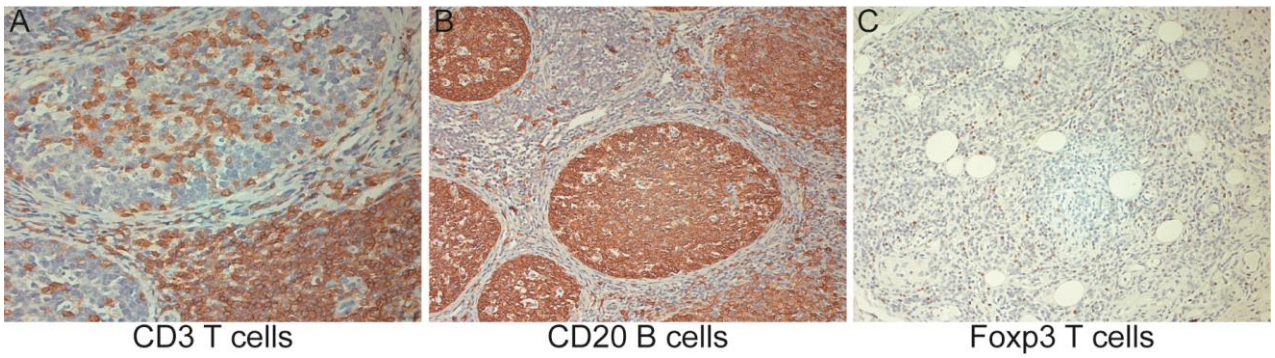

Figure 6: Immunohistochemistry staining for CD3 T cells, CD20 B cells and Foxp3 regulatory T cells in the skin component of heterotopic hind limb transplant recipients.

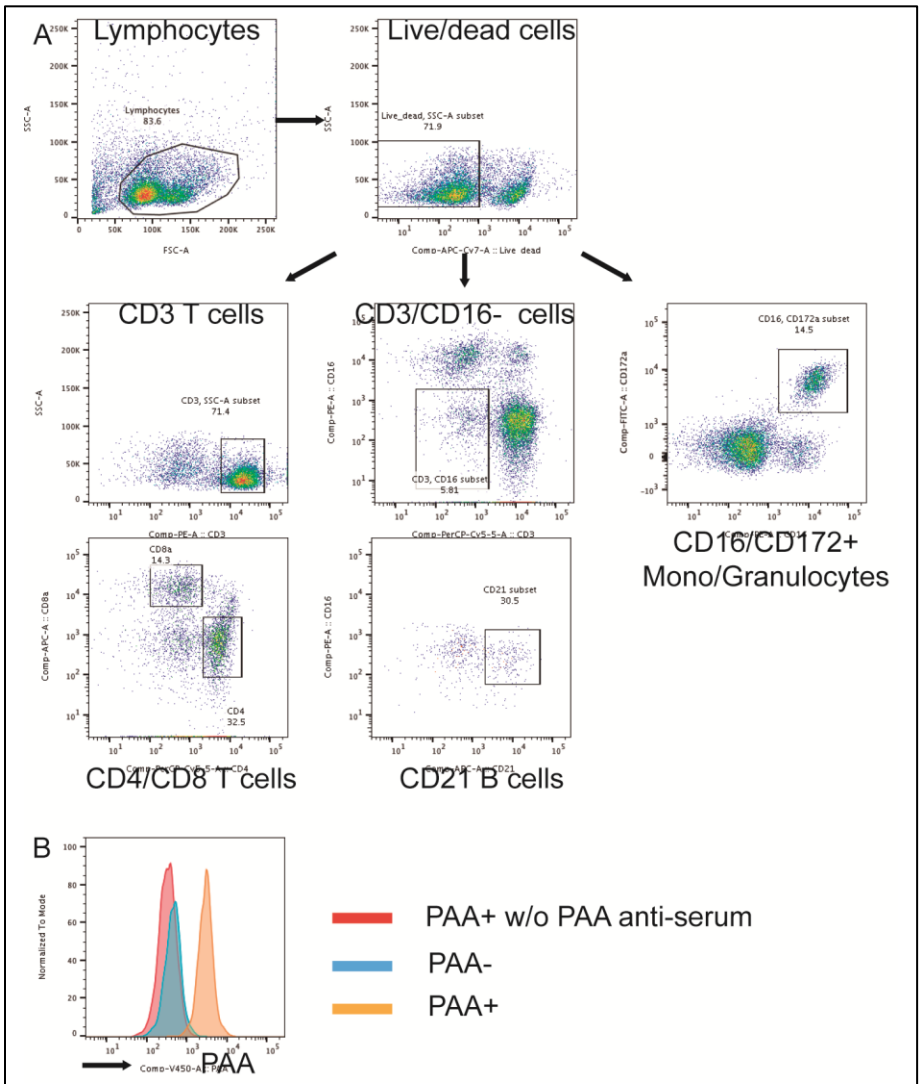

Figure 7: Representative gating strategies for PBMCs' repopulation and PAA-based chimerism analysis.
and donor derived PAA^+ cell detection

Significant results or key outcomes

The investigators successfully performed initial control experiments in unsensitized animals applying the proposed induction and maintenance immunosuppressive regimen to assess its safety and feasibility for maintaining VCA allograft survival in this model. Unfortunately, due to the lack of available animals from the breeder at MGH/Columbia no further transplants could be performed (see detailed explanation below).

Given the lack of available animals for transplant the investigators optimized *in vitro* assays and methodologies such as mixed lymphocyte reaction, immunohistochemistry staining for T, B and regulatory cells, detection of donor specific antibodies, detection of PBMCs' repopulation and donor derived chimerism.

Other achievements

C. Training and Professional Development

The completion of Task 1 of the SOW has provided the PI's with the opportunity to solidify the training of the involved research fellows with regards to optimizing both microsurgical technique (swine heterotopic hind limb transplantation model) as well as advanced *in-vitro* assays for the assessment of donor-specific response.

D. Result Dissemination

Nothing to report

E. Future plan

The plan for the upcoming year is to complete the transplants proposed in the SOW for Subtasks 1 and 2 as soon as additional animals will be available from the breeder in an expedited fashion and to continue with the experiments as outlined by the SOW for year 2.

4. IMPACT

A. Impact on the Development of the Principal Discipline(s) of the Project

The development of specifically designed animal models as proposed in this study will be a prerequisite to pave the way to the acquisition of the lacking DSA and AMR data indispensable to the further advancement of field of VCA. The insights gained from this project will lead to a better understanding of the molecular and pathological sequelae of DSA and AMR in VCA. This will bring us closer to developing specific, targeted, and clinically applicable treatment modalities for AMR. In particular, the use of autologous HSCT as a novel, rapidly translatable desensitization approach will have a significant impact on our discipline and will allow us to successfully perform VCA in highly sensitized patients who otherwise would be excluded as candidates for transplantation.

B. Impact on Other Disciplines

A better understanding of DSA and AMR in VCA, along with the development of clinically applicable desensitization protocols, will not only contribute greatly to the advancement of the field of reconstructive transplantation but also be applicable to other types of solid organ transplantation to enable desensitization in a cadaveric donor setting.

C. Impact on Technology Transfer

Nothing to Report

D. Impact on Society beyond Science and Technology

Nothing to Report

5. CHANGES/PROBLEMS

Nothing to Report

A. Changes in Approach and Reasons for Change

Nothing to Report

B. Actual or Anticipated Problems or Delays and Actions or Plans to Resolve Them

We have been experiencing significant delays in getting started with Major Task 1 and 2 due to limited animal availability from the breeder at Massachusetts General Hospital (MGH). This has been due to the fact that personnel and subsequently large parts of the breeding herd have been relocating from MGH to Columbia University. However, over the past couple of months, we have made significant progress in defining the source and origin of swine from MGH/Columbia University and initiated specific breeding efforts to meet our demands for this study. As stated above, initiatives are underway to assure continued access to the animals. Based on several phone conversations with Dr. David Sachs and the staff from the breeding facility it is expected that animals will be available later this fall.

To carefully address the specific needs of the experiments (SLA type, PAA+, gender, size, age) as outlined by the SOW specific breeding pairs to allow for reproducibility are required and the investigators have been assured that those will be provided for this project. Based on latest conversations the animals required for this project are currently actively bred. As our team has extensive experience with the proposed large animal experiments we don't foresee any additional issues in order to accelerate the pace of the proposed transplants and thereby make up for the experienced delays thus far.

Moreover, kinetics of donor specific antibodies formation and immunohistopathological studies are pending and we are awaiting to collect more samples to process and analyze.

C. Changes that had a Significant Impact on Expenditures

Nothing to Report

D. Significant Changes in Use or Care of Human Subjects, Vertebrate Animals, Biohazards, and/or Select Agents

Nothing to Report

6. PRODUCTS

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

No changes

B. Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period

Nothing to Report

C. Other organizations involved as partners

Nothing to Report